Int J Gynecol Cancer 2000, **10,** 477–487

Prognostic significance of cell cycle proteins and genomic instability in borderline, early and advanced stage ovarian carcinomas

H. BLEGEN*, N. EINHORN†, K. SJÖVALL‡, A. ROSCHKE§, B. M. GHADIMI§, L. M. MCSHANE¶, B. NILSSON*, K. SHAH**, T. RIED§, & G. AUER*

*Departments of *Oncology and Pathology, ‡Gynecology and †Gynecological Oncology, Karolinska Hospital, Stockholm, Sweden; §Genetics Department, Division of Clinical Sciences, and ¶Biometric Research Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and **Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland*

Abstract. Blegen H, Einhorn N, Sjövall K, Roschke A, Ghadimi M, McShane LM, Nilsson B, Shah K, Ried T, Auer G. Prognostic significance of cell cycle proteins and genomic instability in borderline, early and advanced stage ovarian carcinomas. *Int J Gynecol Cancer* 2000;**10:**477–487.

Disturbed cell cycle-regulating checkpoints and impairment of genomic stability are key events during the genesis and progression of malignant tumors. We analyzed 80 epithelial ovarian tumors of benign (*n* = 10) and borderline type (*n* = 18) in addition to carcinomas of early (*n* = 26) and advanced (*n* = 26) stages for the expression of Ki67, cyclin A and cyclin E, $p21^{WAF-1}$, $p27^{KIP-1}$ and p53 and correlated the results with the clinical course. Genomic instability was assessed by DNA ploidy measurements and, in 35 cases, by comparative genomic hybridization. Overexpression of cyclin A and cyclin E was observed in the majority of invasive carcinomas, only rarely in borderline tumors and in none of the benign tumors. Similarly, high expression of p53 together with undetectable p21 or loss of chromosome arm 17p were frequent events only in adenocarcinomas. Both borderline tumors and adenocarcinomas revealed a high number of chromosomal gains and losses. However, regional chromosomal amplifications were found to occur 13 times more frequently in the adenocarcinomas than in the borderline tumors. The expression pattern of low p27 together with high Ki67 was found to be an independent predictor of poor outcome in invasive carcinomas. The results provide a link between disturbed cell cycle regulatory proteins, chromosomal aberrations and survival in ovarian carcinomas.

KEYWORDS: carcinoma, cell cycle, genomic instability, ovary, prognosis.

Ovarian cancer is the leading cause of death among gynecological cancers. About 90% of the ovarian cancers are of epithelial origin. The median age of the patients at the time of diagnosis of ovarian cancer is 63 years. Most cases are sporadic, but 5% occur as an inherited cancer syndrome[1]. Seventy percent of the patients present with advanced stage disease at the time of diagnosis, which contributes to the poor prognosis[2]. The 5-year survival rate for early stage carcinomas is 80%. Patients with advanced disease have a 15% 5-year survival rate.

Borderline (low malignant potential) tumors represent approximately 10% (4–14%) of all ovarian malignancies[3]. In contrast to the truly invasive epithelial

Address correspondence and reprint requests to: Dr. Gert Auer, Department of Oncology and Pathology, CCK R8:04, Karolinska Hospital, SE-171 76 Stockholm, Sweden. Email: Gert.Auer@ks.se.

ovarian carcinomas, the prognosis of the borderline cases is far superior and this is independent of disease stage[3]. Among the early stage borderline cases, the 5-year survival is 93% and among the advanced stages, about 75%. The treatment of epithelial ovarian cancer is primarily surgical, with either complete resection or debulking procedures followed mainly by chemotherapy and, in selected cases, by radiotherapy.

The pronounced differences in the clinical course are the reasons for an ongoing search for diagnostic and prognostic markers to explain the biological behavior of the tumors[4]. Markers such as DNA ploidy[5–7], S-phase fraction[8], proliferative activity (Ki67)[9], p53 status[9,10] and other factors, e.g., HER-2/neu[11], have all been studied with respect to prognostication of ovarian cancers. More recently, the analysis of cell cycle regulatory proteins $p27^{KIP-1}$ and cyclin E suggests their potential as significant prognostic markers for survival in carcinomas of the breast[12], colon[13] and prostate[14], and $p27^{KIP-1}$ was reported as a prognostic marker in ovarian carcinomas[15].

The different cellular events associated with the initiation, progression and metastasis of tumors are still poorly understood. However, one feature of tumor cells is their ability to acquire gene mutations and chromosomal aberrations. Possible causes of this instability include defective cell cycle regulation that can result in premature initiation of DNA replication, defects in the G2/M-control with re-replication of the DNA (polyploidization) or a premature initiation of chromatin condensation and mitosis of incompletely replicated DNA[16,17].

The TP53 suppressor gene regulates the expression of multiple proteins, including p21/WAF-1. This cyclin-dependent kinase inhibitor interacts with the cdc/cdk-cyclin system in the regulation of the cell cycle. P53 is also involved in coordinated centrosome duplication prior to mitosis[18].

Cytogenetic studies performed on cell lines and short-term cultures from primary ovarian tumors as well as on resected tumor material revealed that chromosomal aberrations frequently occur in borderline tumors and invasive carcinomas[19–22]. Also, comparative genomic hybridization (CGH) studies of ovarian tumors showed that the pattern of chromosomal aberrations in defined subgroups of these tumors is nonrandom[23,24].

The aim of the present study was to correlate the expression of different cell cycle regulatory proteins and chromosomal rearrangements with the clinical course and to explore the prognostic impact of these markers in benign tumors and selected cases of borderline, early and advanced invasive carcinomas.

Materials and methods

Patients and samples

At the Department of Pathology, Karolinska Hospital, we analyzed primary epithelial ovarian tumor samples from 80 patients operated and staged according to International Federation of Gynecologic Oncology (FIGO) guidelines and classified according to the World Health Organization. All patients were selected according to tumor stage and survival. The observation time was more than 10 years, and no cases were lost for follow-up.

We selected 18 borderline tumors, 26 early invasive cancers in stage Ia-IIa and 26 advanced cancers in stage IIb-IV. In addition, 10 benign cystadenomas were included. In each of the group of patients with malignant disease, we selected patients with a different clinical course (dead of disease or survival > 10 years). Among the nonsurvivors, one patient had a borderline tumor (stage Ib, survival time 57 months), five were in early stage and 15 were in advanced stage. For a summary of the histopathologic diagnoses, see Table 1.

Treatment

None of the patients received preoperative chemo- or radiotherapy. Of the patients with borderline tumors, 10 did not receive any postoperative treatment, four were irradiated, three received chemotherapy, and one received both irradiation and chemotherapy.

Table 1. Distribution of the histologic types in different groups in 80 ovarian tumors

Number of tumors			
Histologic type	Total	Early stage IA–IIA	Advanced stage IIB–IV
Benign			
Serous	6		
Mucinous	4		
Borderline			
Serous	14	11	3
Mucinous	4	4	0
Invasive cancer			
Serous	21	6	15
Mucinous	3	3	0
Endometroid	18	11	7
Mesonephrioid	8	6	2
Anaplastic	2	0	2

Among the early stage cases, 19 received both irradiation and chemotherapy, one received irradiation only, four received chemotherapy only, and two received no postoperative treatment. Fifteen of the advanced stage cases were treated with both irradiation and chemotherapy and 11 with chemotherapy only. The chemotherapy at different time periods consisted of melphalan only, a combination of melphalan and doxorubicin or a cisplatin-based combination therapy.

Sample preparation

All tumor samples were fixed directly after the operation in 4% phosphate buffered formalin and were paraffin embedded. Ten consecutive sections were prepared from each specimen and used for immunohistochemistry (4 μm), DNA ploidy measurements (8 μm) and tissue microdissection (50 μm). Tumor cell areas were microdissected from two 50 μm sections to obtain samples containing more than 80% tumor cells. The tissue was incubated overnight in 1 M NaSCN at 37°C and washed twice in PBS. The DNA was isolated by standard proteinase-K digestion, phenol-chloroform extraction and ethanol precipitation. All samples were coded, and the analyses were performed in a blinded fashion.

Immunohistochemistry

All slides were deparaffinized with xylene, rehydrated and microwaved at 500 W for 2 × 5 min in 10 mM citrate buffer, pH6.0. Sections were blocked with 3% hydrogen peroxide in methanol, followed by normal horse serum (1:20 dilution) in 0.1 M PBS, pH 6.0 and then incubated overnight with the different antibodies diluted (see below) in 1% (wt/vol) bovine serum albumin and visualized by standard avidin biotin-peroxidase complex technique (Vector Laboratories, Burlingame, CA). The antibodies used were: NCL-p27, dilution 1:100 (Novocastra Laboratories LTD, Newcastle upon Tyne, UK); MIB 1 (Immunotech S.A., Marseille, France) dilution 1:150; DO1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) dilution 1:100; WAF1 (Ab-1) (Oncogene Science, Cambridge, MA), dilution 1:15; NCL-cyclin A (Novocastra), dilution 1:100; and NCL-cyclin E (Novocastra), dilution 1:40.

All slides were coded and scored independently by two pathologists (G.A. and H.B.) and 10 HPF (highest staining intensity areas) of tumor cells were scored according to a previous study[12]. All antibodies used are raised against nuclear antigens, and only cells with distinct nuclear staining were considered positive. All sections contained both normal and tumor cells, which allowed comparison of immunohistochemical staining of benign and malignant cells.

DNA ploidy measurements

The amount of total nuclear DNA was measured by image cytometry on Feulgen- stained histological slides 8 μm thick as described[25]. The DNA histograms were interpreted according to a modified subjective method. The normal control cells were given the value 2c, denoting the normal diploid DNA content, and all tumor cell DNA values were expressed in relation to that. The histograms were divided into two groups. Cases with a major peak near the 2c region (1.8c–2.2c) and less than 10% of the cells exceeding 2.5c were classified as diploid (even if CGH revealed chromosomal copy number alteration). DNA profiles with a stemline outside the diploid region and distinctly scattered DNA values exceeding the tetraploid region (3.8c-4.2c) were classified as aneuploid.

Comparative genomic hybridization

Normal control DNA was prepared from human female lymphocyte DNA and labeled in a standard nick-translation reaction substituting dTTP with digoxigenin-11dUTP. Tumor DNA was labeled with biotin-16dUTP (Boehringer Mannheim). For CGH, 500 ng of tumor DNA and 500 ng of the reference DNA were ethanol precipitated in the presence of 10 μg salmon sperm DNA and 40 μg of the Cot-1 fraction of human DNA (Gibco BRL, Gaithersburg, MD). The probe was dried and resuspended in 10 μl hybridization solution (50% formamide, 2× SSC, 10% dextran sulfate), denatured for 5 min and preannealed for 60 min at 37°C. For reliable karyotyping, human centromeric probes for chromosome 4, 8, 14, 20, 22 and X were directly labeled by PCR substituting dTTP with Cy-5–labeled dUTP (Boehringer Mannheim) and cohybridized with the genomic probes. The probe cocktail was hybridized to normal, human female metaphase chromosomes. The metaphase chromosomes were denatured separately (70% formamide, 2× SSC for 2 min at 75°C), and dehydrated through an ethanol series (70–90–99%). Hybridization took place under a coverslip for 2 days at 37°C.

Posthybridization and detection was performed as described elsewhere[26–27]. The chromosomes were counterstained with 4–6-diamidino-2-phenylidole for chromosome identification (DAPI-banding) and mounted in 1,4-phenylene-diamine.

Microscopy and digital image analysis

Individual metaphases were karyotyped and typically 10 metaphases were analyzed for each tumor case. Three gray scale images of the chromosomes were acquired using a Leica DMRBE epifluorescence microscope and the Cyto Vision program (Applied Imaging Corporation, Newcastle upon Tyne, UK). The fluorescent intensity profiles (red and green) were calculated and the threshold values used were 0.75 (loss) and 1.25 (gain).

The average number of chromosomal copy aberrations (ANCA) was calculated by adding all chromosomal events for different groups divided by the number of cases in each group. The average number of regional amplifications (ANRA) was calculated by adding all regional amplifications for different groups divided by the number of cases in each group.

Statistics

Univariate and multivariate survival analysis were conducted using Cox proportional hazards regression models with testing performed likelihood ratio methods. Immunohistochemical markers were investigated for the expression pattern within and between tumor groups as well as in correlation with chromosomal rearrangements and survival. Between-group comparisons of categorized marker levels were conducted using Fisher's exact test. Patient treatment decisions were made without knowledge of the immunohistochemical markers or chromosomal rearrangements, and survival analyses were not adjusted for treatment. However, a small difference in survival might be expected due to the different treatment modalities. No adjustment was made for multiple comparisons due to the large number of comparisons and the likelihood of correlations, which would make adjustments impractical. As such, the findings require confirmation in independent clinical studies.

Results

DNA ploidy

All benign cystadenomas were diploid. Due to a high tumor cell density, six borderline tumors, two early stage cancers and four advanced stage cancers could not be reliably evaluated[25]. In the advanced stage tumors, 16 were aneuploid and six diploid. In the early stage tumors, 15 were aneuploid and nine diploid. In the borderline group, three were aneuploid and nine diploid. An increase in diploid vs. aneuploid tumors was significant when borderline tumors were compared to invasive cancers ($P = 0.02$) but not between early and advanced carcinomas (Table 2). There was marginal significance between survival and ploidy in patients with invasive tumors ($P = 0.059$) (Table 3).

Immunohistochemistry

Ki67

All benign cystadenomas revealed a low proliferative activity (< 20% positive cells). Only one case among the borderline tumors showed a high level in proliferative activity (> 20% positive cells). This patient subsequently died from the disease. In the early stage carcinomas, 13 of 26 cases showed a high Ki67 staining, whereas in the advanced stages, 18 of 26 tumors revealed a high level (≥ 20% positive cells). In univariate analysis, a significant difference between borderline and invasive carcinomas was seen in Ki67 expression ($P = 0.003$ and $P < 0.0001$ for borderline vs. early and advanced carcinomas, respectively) (Table 2). There was no statistical difference in proliferative activity between early and advance stage carcinomas ($P = 0.26$). For the two invasive groups together, there was a significant difference in survival between those with low vs. high proliferation ($P = 0.016$, Table 3).

Of patients who had tumors with a low number of Ki67 positive cells, 92% survived at least 10 years compared with 47% of those who had high Ki67 levels. In the group of patients with early stage disease, there was an equal number of tumors with high and low Ki67, and survival was not correlated to proliferative activity. However, in the group of patients with advanced stage tumors, we observed a correlation of increased Ki67 expression and short survival.

$P27^{KIP-1}$

All benign cystadenomas showed low levels of p27 (< 50% positive cells). In the borderline group, six tumors had a low p27 expression, including the only patient who died from the disease. There was no significant difference in the distribution of p27 staining patterns in borderline and early invasive cases. However, there was a significant difference in the expression pattern between borderline and advanced carcinomas as well as between early and advanced carcinomas ($P = 0.025$, Table 2). A significant correlation between low p27

Table 2. Expression of biological markers (Fisher's exact test) in benign cystadenomas, borderline tumors and early and advanced stage carcinomas of the ovary

Markers	Benign no. of cases (%)	Borderline no. of cases (%)	Early stage no. of cases (%)	Advanced stage no. of cases (%)
DNA ploidy[b,d]				
Diploid	10 (100)	9 (84)	9 (56)	6 (35)
Aneuploid	(0)	3 (16)	15 (44)	16 (65)
Ki67[a,b,d]				
<20%	10 (100)	17 (94)	13 (50)	8 (31)
≥20%	(0)	1 (6)	13 (50)	18 (69)
P27[c]				
<50%	10 (100)	6 (33)	8 (31)	17 (65)
≥50%	(0)	12 (67)	18 (69)	9 (35)
Cyclin A[a,b]				
<5%	10 (100)	18 (100)	10 (38)	4 (15)
≥5%	(0)	(0)	16 (62)	22 (85)
Cyclin E[a]				
<10%	10 (100)	14 (78)	10 (38)	13 (50)
≥10%	(0)	4 (22)	16 (62)	13 (50)
P53[a,b]				
<10%	10 (100)	18 (100)	19 (73)	14 (54)
≥10%	(0)	(0)	7 (27)	12 (46)
P21				
<20%	5 (50)	4 (22)	17 (35)	12 (46)
≥20%	5 (50)	14 (78)	9 (65)	14 (54)

[a]Significance between borderline and early stage tumors.
[b]Significance between borderline and advanced stage tumors.
[c]Significance between early and advanced stage tumors.
[d]Significance between borderline and invasive tumors.
In column 1, the percentage of positive-staining tumor cells and the borders between low and high are indicated.

and survival in invasive tumors was found (P = 0.0001, Table 3).

Cyclin A and cyclin E

All benign cystadenomas and borderline tumors revealed low cyclin A levels (< 5% positive cells). Patients with borderline and early stage carcinomas (P = 0.0001) and borderline and advanced stage carcinomas (P = 0.0001), however, revealed a significant difference. This difference was not detected when early and advanced cases were compared (P = 0.12, Table 2). Furthermore, no significant association was found between survival and increased cyclin A expression (≥5% positive cells) in the group of invasive tumors (P = 0.33, Table 3).

All benign cystadenomas had a low cyclin E expression (<10% positive cells). There was a significant difference in cyclin E expression pattern between borderline and early stage cases (P = 0.0281), but no significant difference when early and advanced stage tumors were compared (Table 2, Fig. 1). We did not find a significant association between survival and in-

Table 3. Univariate survival analysis (Cox proportional hazards regression of biological markers in invasive ovarian carcinomas (early and advanced groups)

Markers	Unadjusted relative hazard (95% CI)	LR[a] P-values
DNA ploidy		
Diploid	1.00	
Aneuploid	2.80 (0.80, 9.74)	0.074
Ki67		
<20%	1.00	
≥20%	4.76 (1.13, 10.19)	0.016
p27		
<50%	1.00	
≥50%	0.14 (0.048, 0.44)	0.0001
Cyclin A		
<5%	1.00	
≥5%	1.67 (0.56, 5.01)	0.33
Cyclin E		
<10%	1.00	
≥10%	0.76 (0.32, 1.83)	0.54
p53		
<10%	1.00	
≥10%	1.33 (0.54, 3.26)	0.54
p21		
<20%	1.00	
≥20%	0.80 (0.33, 1.93)	0.62

[a]Likelihood ratio.

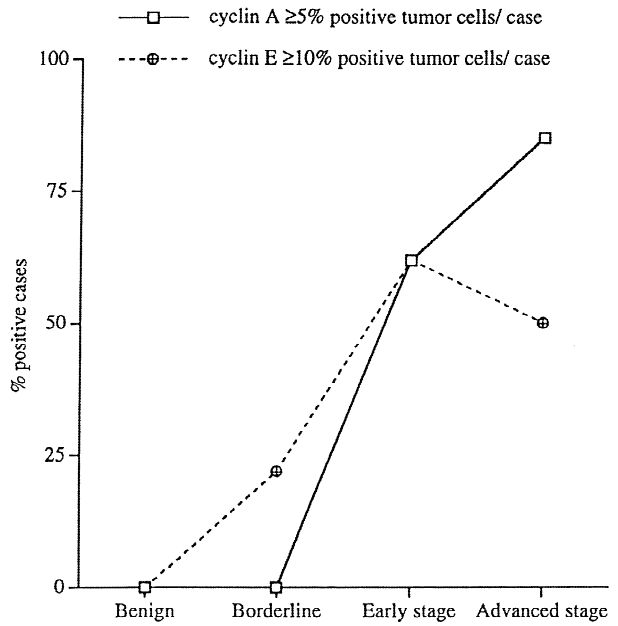

Fig. 1. Expression of the cell cycle regulatory proteins cyclin A and cyclin E detected by immunohistochemistry in different groups of ovarian tumors.

creased frequency of cyclin E expression (≥10% positive cells) in early and advanced carcnomas (P = 0.54, Table 3).

P53 and p21

Immunohistochemical staining in benign cystadenomas or borderline tumors with antibody against p53 (DO1) revealed a negative staining pattern; however, in 50% of the benign and 78% of the borderline cases, p21 immune staining revealed an increased level. In contrast, seven cases in the early stage group and 12 cases in the advanced stage group showed increased expression of p53 (≥10% positive cells). Of these nineteen cases, eight cases revealed undetectable levels of p21. Regarding high p53 expression or high p53 expression associated with undetectable p21 levels, there was no statistically significant difference between the early and advanced stage tumors (Table 2). No significant correlation was found between increased p53 expression with or without undetectable p21 levels and survival in this study.

Uni- and multivariate analysis

Of the potential predictors of disease progression, i.e., histologic type, tumor grade and stage, DNA ploidy, and the expression levels of cyclin A, cyclin E, p21, p53, p27 and Ki67 that were analyzed using the Cox multiple regression model, p27 and Ki67 were the only markers found to be significant and independent markers (Table 3). A reduced model containing only p27 and Ki67 was fit (Table 4) When p27 and Ki67 were combined to jointly form four possible risk groups, high expression of Ki67 together with low expression of p27 gave a highly significant (P = 0.0018) relative risk of death (relative risk, 10.8; 95% confidence interval, 2.4–48.5) as compared to low Ki67 together with high p27 (Fig. 2).

Comparative genomic hybridization

Comparative genomic hybridizataion revealed a different pattern of chromosomal rearrangements between borderline and early and advanced stages of invasive tumors (Fig. 3). The average number of copy alterations increased between all groups from borderline to early and advanced stage adenocarcinomas. The borderline tumors had an ANCA value of 8.0. Diploid invasive carcinomas had an ANCA value of 6.8 and aneuploid cancers had an ANCA value of 16.2. Concerning the average number of regional amplifications (ANRA values), there was a significant difference between the groups. Borderline tumors, early stage carcinomas and advanced stage carcinomas had ANRA values of 0.1, 1.1 and 1.2, respectively (overall P-value = 0.03, see also Fig. 4).

A high ANCA value of 19.3 was found for tumors with high p53 expression associated with negative p21 levels. This high ANCA value was also found in grade III tumors (19.3), irrespective of tumor type or stage. In both the group of grade III tumors and in the group of high-p53/negative-p21 tumors, 92% were aneuploid. The tumors overexpressing cyclin A and cyclin E had an ANCA value of 16.4, and tumors with normal levels of cyclin A and cyclin E had an ANCA value of 7.6. Tumors with high Ki67/low p27 staining pattern had an ANCA value of 14.9, whereas tumors with low Ki67/high p27 had an ANCA value of 7.5. When tumors with loss of chromosome arm 17p were added to the group of tumors with high p53 expression and negative p21 levels, the ANCA value was 15.2,

Table 4. Cox multivariate analysis with respect to disease-specific survival in invasive ovarian carcinomas (early and advanced stage)

Markers	Relative risk (95% CI)	P-value LR[a]
Ki67		
<20%	1.00	
≥20%	3.4 (1.12, 10.26)	0.017
$p27^{KIP-1}$		
<50%	1.00	
≥50%	0.14 (0.05, 0.43)	0.0001

[a]Likelihood ratio.

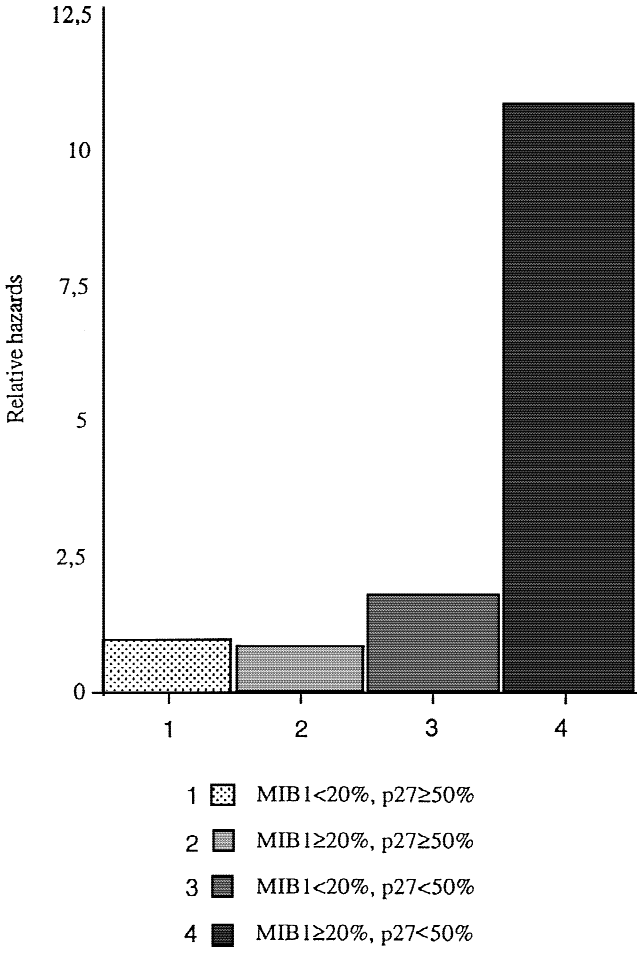

Fig. 2. Model-based values of relative hazards from Cox modified multiple regression with respect to disease-specific survival (dead from disease or alive > 10 years) in invasive ovarian carcinomas. Immunostaining, Ki67 and p27 stratified into four groups (high vs. low values).

whereas p53 expression negative tumors (i.e., no loss of 17p and negative IHC staining) had an ANCA value of 6.0 (Fig. 5). In six of the 16 cases that were positive for cyclin A and cyclin E expression, we observed increased p53 expression and negative p21 levels.

The tumors with increased frequency of cells positive for cyclin A and cyclin E expression had an ANRA value of 1.8, whereas tumors with normal levels of cyclin A and cyclin E had an ANRA value of 0.3. Tumors with high Ki67 and low p27 staining patterns had an ANRA value of 1.0, whereas tumors with a low Ki67 and high p27 had an ANRA value of 0.5. The tumors with high p53 expression and negative p21 staining patterns or tumors with a loss of chromosomes arm 17p had an ANRA value of 1.5, whereas p53 negative tumors had an ANRA value of 0.06 (Fig. 6).

Diploid invasive carcinomas had an ANCA value of 6.8 and an ANRA value of 0.2; the aneuploid cancers had an ANCA value of 16.2 and ANRA value of 1.6. Forty-two percent of the chromosomal aberrations in 11 borderline tumors consisted of copy number changes of entire chromosomes. This number was reduced to 19.3% and 10.9% for early and advanced stage carcinomas, respectively. The most frequent aberrations seen in all tumors were gains of chromosome arms 8q (52%), 3q (44%), 5p (32%), Xq (32%), and 12p (28%) and losses that mapped to 22q (28%) and 17p (32%). A statistically significant association was found between gain of chromosome 7 and early stage disease ($P = 0.0019$) and also between loss of chromosome arm 13q and high frequency of tumor cells expressing cyclin A ($P = 0.008$).

Comparative genomic hybridization results were obtained from 25 invasive carcinomas. Of these, irrespective of stage and treatment, 16 patients survived more than 10 years, and nine died. The ANCA value for the survivors was 12.1, and for the nonsurvivors, the value was 14.2. No statistical association was found between ANCA or ANRA and survival. Gain of chromosome arm 3q was found in 89% of the cases in the group of nonsurvivors but only in 50% of the survivors. The survivors showed loss of 4q in 50% of the cases and a gain of 7q in 62%, but these aberrations occurred in only 22% (– 4q) and in 11% (+ 7q) of the nonsurvivors. Loss of 17p was seen in 44% of the survivors and in 33% of the nonsurvivors.

Discussion

Previously established prognostic factors in ovarian cancer were stage, age, histologic type, grading and postoperative residual tumor. These were the prognostic factors that were instrumental in the strategy of therapy. Unfortunately those factors are not always strong enough to recognize borderline tumors from invasive carcinomas or to differentiate high and low risk patients, especially in early stages. There is an ongoing discussion how to treat patients in early stage disease, and there is a clear trend to avoid adjuvant therapy as well as extensive surgery in those cases. Many attempts have been made to introduce molecular markers in order to differentiate between low and high risk ovarian cancers. DNA ploidy has been the first molecular prognostic factor that has been investigated in this tumor group. Subsequently, p53, p21, Ki67, p27 and HER-2/neu were investigated with the same purpose.

We have therefore investigated the relationship between genomic instability, the expression of cell cycle regulatory proteins and the outcome of patients with ovarian epithelial tumors. We were specifically inter-

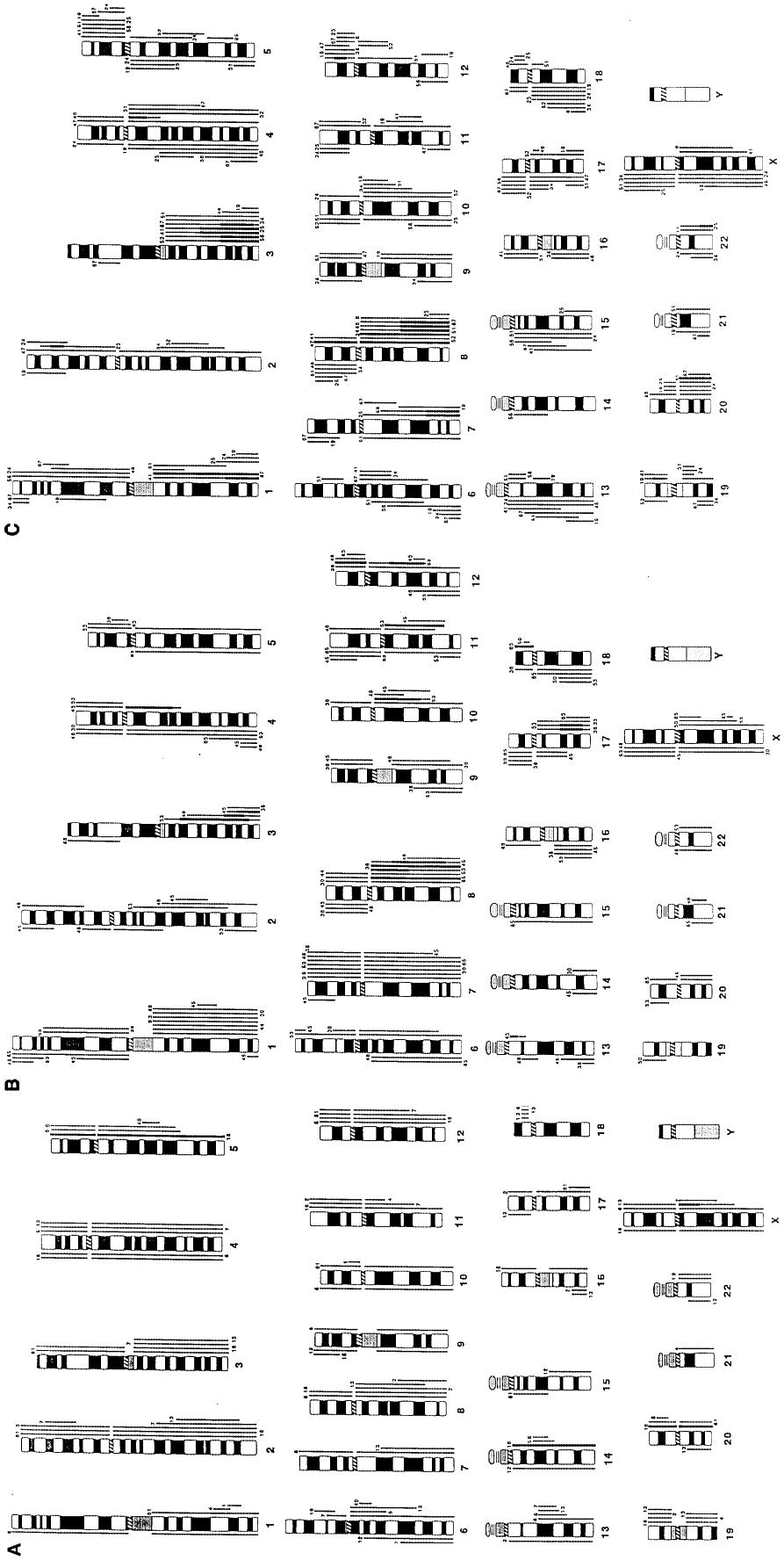

Fig. 3. Karyogram of chromosomal gains and losses in: (A) borderline tumors ($n = 11$); in (B) early stage ovarian carcinomas ($n = 11$); and in (C) advanced stage ovarian carcinomas ($n = 13$). Bars on the right side of the chromosome ideogram indicate gains and bars on the left side indicate loss of genetic material. Solid bars denote high level copy number increases (amplifications). The number above/below the bar indicates the case number.

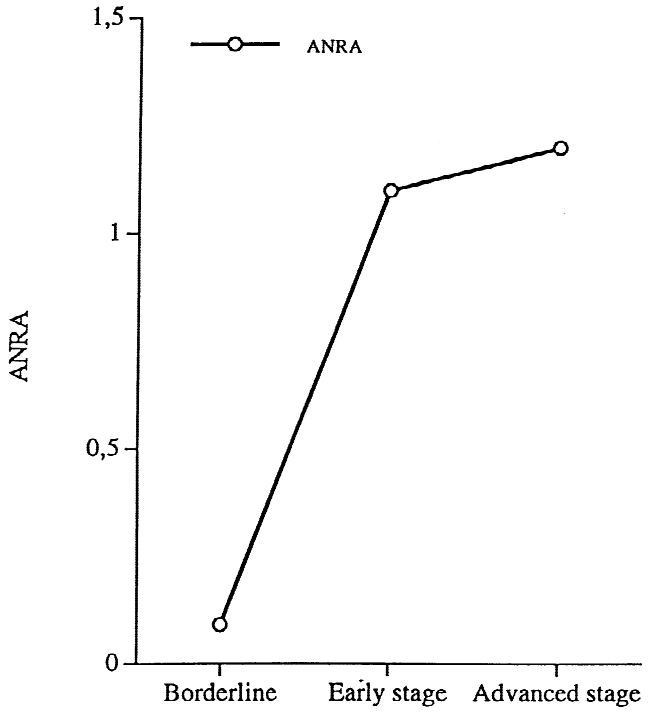

Fig. 4. The average number of regional amplifications (ANRA) detected by comparative genomic hybridization in different groups of ovarian tumors.

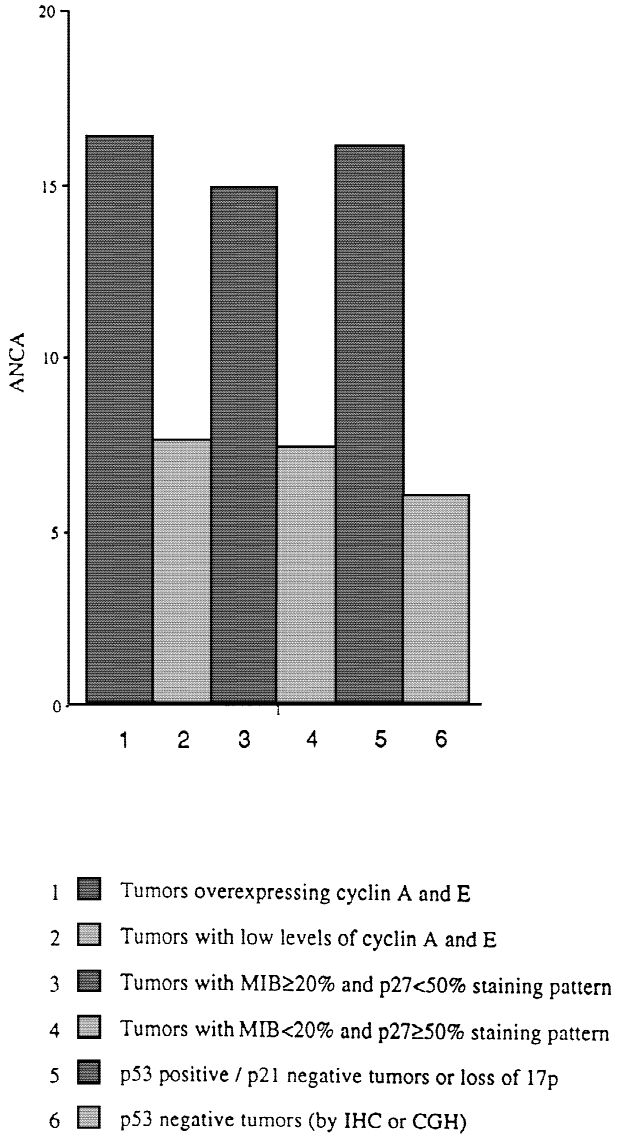

Fig. 5. The correlation between the average number of chromosomal aberrations (ANCA) in relation to the expression of different cell cycle regulatory proteins.

ested to explore whether disturbed cell cycle control and impaired genomic integrity coincide with histopathologically defined benign cystadenomas, borderline tumors and invasive adenocarcinomas. We also wanted to explore if these aberrations can explain the differences in the clinical behavior within these histopathologic entities in general and within the group of malignant tumors in particular.

The group of benign cystadenomas was homogenous for the cell cycle regulatory proteins and crude DNA ploidy. All tumors revealed a diploid DNA histogram and normal levels of cyclin E, cyclin A, p53 and p21. Also, low expression of p27 was found in the benign tumors. One possible explanation for these findings is that these tumor cells are terminally differentiated.

The borderline tumors seemed to be a heterogeneous group. The majority of the tumors resembled benign cystadenomas; however, some revealed a pattern more similar to the one observed in adenocarcinomas. In this study of epithelial ovarian tumors, we found that borderline tumors frequently express cyclin A and cyclin E at normal (i.e., low) levels, have normal p53 and p21 levels, are mostly diploid and have a favorable clinical outcome. Despite seemingly normal cell cycle regulation, these tumors do have chromosomal aberrations, and this is reflected by an ANCA value of 8 (similar to the one observed in diploid invasive carcinomas, where the ANCA value is 6.8). However, almost 50% of these aberrations consisted of whole chromosome gains and losses, which would indicate that chromosome segregation errors are early events. Only one high-level copy number increase was observed (ANRA value of 0.1) in a patient who rapidly succumbed to the disease. The lack of high-level copy number increases (amplifications) was found to be the most pronounced difference detected by CGH between borderline and invasive tumors (Fig. 4).

In summary, the present findings suggest that borderline tumors are distinctly different biologically

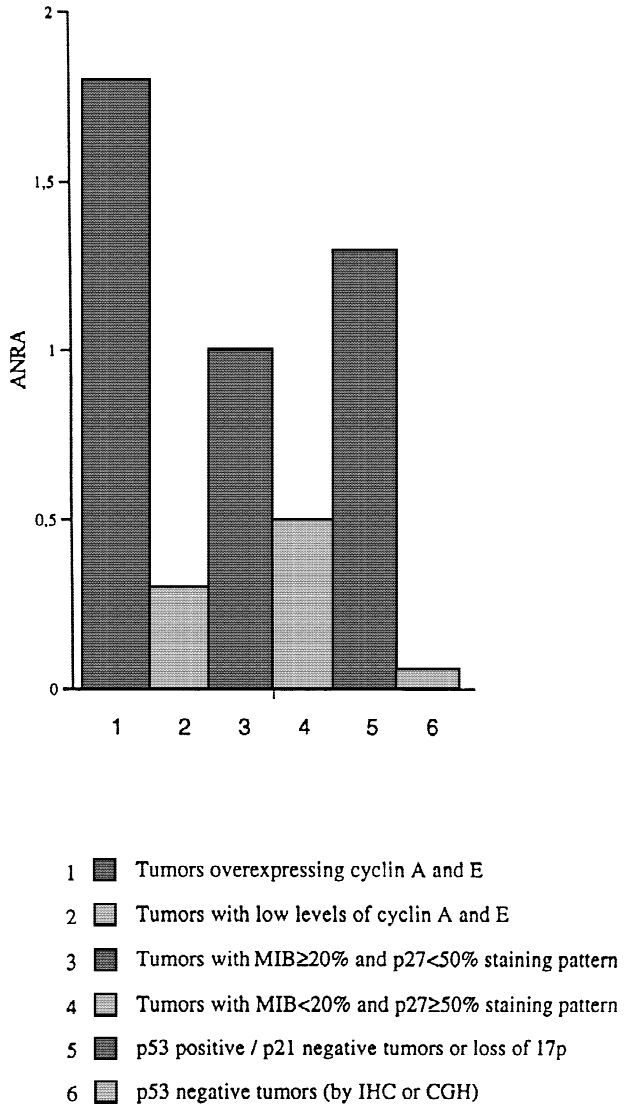

Fig. 6. The correlation between the average number of regional amplifications (ANRA) in relation to the expression of different cell cycle regulatory proteins.

from invasive ovarian carcinomas and that biological markers can contribute to discriminate borderline from invasive variants.

Invasive carcinomas frequently overexpress cyclin A or cyclin E, or both, and show signs of p53 mutations, high proliferative activity with reduced p27 inhibitor activity and aneuploidy. On average, invasive carcinomas have an ANCA value of 13 and an ANRA value of 1.3, indicating a high grade of genomic instability. The mapped genomic amplifications frequently involve regions harboring oncogenes[28].

For all ovarian tumors, high ANCA values (19.3) were found in tumors with high p53 expression and nondetectable p21 levels. Ninety-two percent of these tumors were aneuploid. Cases with high expression of cyclin A and cyclin E had an ANCA of 16.4 and this group of tumors also had a high ANRA value 1.8 (Fig. 4). This finding is reasonable given that DNA replication is dependent on normal levels and timely expression of cyclin A and cyclin E. Abnormal cyclin A and cyclin E levels possibly result in premature DNA replication and re-replication which can result in gene amplification. This could explain the high ANRA value, which also is supported by two recent reports[29,30].

Highly interesting is the strong correlation between elevated Ki67 levels together with low $p27^{KIP-1}$ activity and survival. Our observation suggests that the combined analysis of Ki67 and $p27^{KIP-1}$ warrant explorations in larger clinical studies for improved prognostication in ovarian cancers.

Acknowledgments

We are grateful to Amy Peng for statistical programming and to Ulla Aspenblad, Birgitta Sundelin and Inga Maurin for technical assistance and Veronique Bruniquel for manuscript preparation. This work was supported by the Swedish Cancer Society and the Cancer Society in Stockholm and by grants from the Swedish Medical Research Foundation. B. Michael Ghadimi was supported by the Deutsche Krebshilfe.

References

1 Schildkraut JM, Thompson WD. Familial ovarian cancer: a population-based case-control study. *Am J Epidemiol* 1988;**128:**456–66.
2 Phyllis A, Tony T, Sherry B. Cancer statistics. *CA Cancer J Clin* 1995;**45:**8–30.
3 Pecorelli S. FIGO annual report on the results of treatment in gynecological cancer. *J Epidemiol Biostat* 1998;3:75–102.
4 Birrer MJ. Discoveries in the cell cycle and ovarian cancer. *Gynecol Oncol* 1997;**64:**187–8.
5 Tropé C, Kaern J. Prognosis and management of borderline tumours of the ovary. *Curr Opin Obstet Gynecol* 1996;**8:**12–6.
6 Auer G, Einhorn N, Nilsson B, Silfverswärd C, Sjövall K. Biological malignancy grading in early-stage ovarian carcinoma. *Acta Oncol* 1996;**35:**93–8.
7 Reles AE, Gee C, Schellschmidt I *et al.* Prognostic significance of DNA content and S-phase fraction in epithelial ovarian carcinomas analyzed by image cytometry. *Gynecol Oncol* 1998;**71:**3–13.
8 Kaern J, Tropé C, Kristensen GB, Pettersen EO. Flow cytometric DNA ploidy and S-phase heterogeneity in advanced ovarian carcinoma. *Cancer* 1994;**73:**1870–7.
9 Viale G, Maisoneuve P, Bonoldi E *et al.* The combined evaluation of p53 accumulation and of Ki-67 (MIB1) labelling index provides independent information on overall survival of ovarian carcinoma patients. *Ann Oncol* 1997;**8:**469–76.

10 Bosari S, Viale G, Radaelli U, Bossi P, Bonoldi E, Coggi G. P53 accumulation in ovarian carcinomas and its prognostic implications. *Hum Pathol* 1993;**24:**1175–9.
11 Slamon DJ, Godolphin W, Jones LA *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;**244:**707–12.
12 Porter PL, Malone KE, Heagerty PJ *et al.* Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breats cancer patients. *Nat Med* 1997;**3:**222–5.
13 Loda M, Cukor B, Tam SW *et al.* Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* 1997;**3:**231–4.
14 Yang RM, Naitoh J, Murphy M *et al.* Low p27 expression predicts poor disease-free survival in patients with prostate cancer. *J Urol* 1998;**159:**941–5.
15 Newcomb EW, Sosnow M, Demopoulos RI, Zeleniuch-Jacquotte A, Sorich J, Speyer JL. Expression of the cell cycle inhibitor p27KIP1 is a new prognostic marker associated with survival in epithelial ovarian tumors. *Am J Pathol* 1999;**154:**119–25.
16 Paulowich AG, Toczyski DDP, Hartwell LH. When checkpoints fail. *Cell* 1997;**88:**315–21.
17 Nojima H. Cell cycle checkpoints, chromosome stability and the progression of cancer. *Hum Cell* 1997;**10:**221–30.
18 Fukasawa K, Choi T, Kuriyama R, Rulong S, Vande Woude GF. Abnormal centrosome amplification in the absence of p53. *Science* 1996;**271:**1744–7.
19 Pejovic T, Iosif CS, Mitelman F, Heim S. Karyotypic characteristics of borderline malignant tumors of the ovary: trisomy 12, trisomy 7, and r(1) as nonrandom features. *Cancer Genet Cytogenet* 1996;**92:**95–8.
20 Pejovic T, Heim S, Orndal C *et al.* Simple numerical chromosome aberrations in well-differentiated malignant epithelial tumors. *Cancer Genet Cytogenet* 1990;**49:**95–101.
21 Pejovic T, Heim S, Mandahl H *et al.* Chromosome aberrations in 35 primary ovarian carcinomas. *Genes Chromosomes Cancer* 1992;**4:**58–68.
22 Thompson FH, Liu Y, Emerson J *et al.* Simple numeric abnormalities as primary karyotype changes in ovarian carcinoma. *Genes Chromosomes Cancer* 1994;**10:**262–6.
23 Iwabuchi H, Sakamoto M, Sakunaga H *et al.* Genetic analysis of benign, low-grade, and high-grade ovarian tumors. *Cancer Res* 1995;**55:**6172–80.
24 Sonoda G, Palazzo J, du Manoir S *et al*. Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q13 in human ovarian carcinomas. *Genes Chromosomes Cancer* 1997;**20:**320–8.
25 Steinbeck RG. Mitotic Failure and Genomic Instability in Benign, Premalignant and Malignant Human Tissues Thesis. Stockholm: Karolinska Institute; 1998.
26 Ried T, Baldini A, Rand TC, Ward DC. Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital imaging microscopy. *Proc Natl Acad Sci USA* 1992;**89:**1388–92.
27 Ried T, Heselmeyer-Haddad K, Blegen H, Schrock E, Auer G. Genomic changes defining the genesis, progression, and malignancy potential in solid human tumors: a phenotype/genotype correlation. *Genes Chromosomes Cancer* 1999;**25:**195–204.
28 Hesketh R. The Oncogene FactsBook. London: Academic Press; 1995.
29 Nielsen NH, Emdin SO, Cajander J, Landberg G. Deregulation of cyclin E and D1 in breast cancer is associated with inactivation of the retinoblastoma protein. *Oncogene* 1997;**14:**295–304.
30 Gray-Bablin J, Zalvide J, Fox MP *et al.* Cyclin E, a redundant cyclin in breast cancer. *Proc Natl Acad Sci USA* 1996;**93:15215-20.**

Accepted for publication September 22, 2000.